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Reduced Connexin43 Expression in High-Grade Human Brain Glioma Cells

RUO-PAN HUANG, MD, PhD,[1]* MOHAMMAD Z. HOSSAIN, PhD,[1] ANIL SEHGAL, PhD,[2] AND ALTON L. BOYNTON, PhD[1]

[1]*Molecular Medicine, Northwest Hospital, Seattle, Washington*
[2]*Pacific Northwest Cancer Foundation, Northwest Hospital, Seattle, Washington*

Background and Objectives: Connexin43 (cx43), a gap junction protein, is implicated in the suppression of tumor cell growth. Numerous cancer cells show a reduction or loss of cx43 expression compared to their normal counterparts. Our previous studies suggest that cx43 expression is decreased in a variety of human brain tumor cell lines. To further investigate the role of cx43 in the development of human gliomas, we performed the present study on human glioma grades I–IV.
Methods: Immunohistochemistry was performed on paraffin-embedded tissue sections of 18 human gliomas to analyze the expression levels of cx43 in different stages of human gliomas.
Results: High levels of cx43 were observed in all normal brain tissue and in glioma grades I and II. In contrast, the expression of cx43 was very weak in grade III gliomas and almost undetectable in grade IV gliomas.
Conclusions: Our data support the hypothesis that reduction of cx43 is involved in the progression of human gliomas.
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KEY WORDS: **connexin43; gap junction communication; gliobastoma; tumor suppressor; immunohistochemistry**

INTRODUCTION

Gap junction communication is involved in growth control [1]. Gap junctions are composed of a family of evolutionarily conserved integral plasma membrane proteins termed connexins (cxs), which allow the passive intercellular exchange of small molecules and ions up to 1,000 daltons [2]. At least 12 members of the cx protein family are known [3], with cx43 having the most widely expressed cx, particularly in brain and heart [1]. Reduced expression of cx43 is observed in numerous cancer cells, such as human prostate cancer cells [4,5] and breast cancer cells [6,7]. Introduction of cx43 into transformed cells resulted in the reversal of transformed phenotypes in several tumor cell lines, including human breast cancer cells [8], rat C6 glioma [9,10], transformed dog kidney epithelial cells [11], human rhabdomyosarcoma [12], and human glioblastoma [13]. Furthermore, established cx43 null cell lines display the phenotypic properties of transformed cells [14].

Gliomas are the most common type of primary brain tumor, yet little information is available on the expression of cx43 in the different stages of gliomas. Here, we present an immunohistochemical analysis of cx43 protein expression in different stages of gliomas. Our data indicate that high-grade gliomas exhibit a profoundly reduced expression of cx43, suggesting a role for diminished expression of cx43 in the progression of human gliomas.

MATERIALS AND METHODS

Immunohistochemistry

Eighteen glioma tissue sections were obtained from the Pathology Department, Northwest Hospital, Seattle, WA. Three of them contained normal adjacent brain tissues. These tissue sections were made from previously formalin-fixed and paraffin-embedded samples. Immu-

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*Correspondence to: Ruo-Pan Huang, MD, PhD, Molecular Medicine, Northwest Hospital, 120 Northgate Plaza Suite 230, Seattle, WA 98125. Fax No.: (206) 368-3009. E-mail: rphuang@nwlink.com
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nohistochemistry was performed as described elsewhere [15]. The avidin-biotin complex (ABC) peroxidase method of staining was employed as described by the manufacturers (ABC, Vector Laboratories, Burlingame, CA). Briefly, 5-μm-thick, formalin-fixed, paraffin-embedded sections were dewaxed and endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Sections were incubated with anti-cx43 antiserum [16] or normal rabbit serum at a dilution of 1:200 overnight at 4°C. The sections were washed in phosphated-buffered saline (PBS), incubated for 30 min with biotinylated goat anti-rabbit immunoglobulin G (IgG) at a 1:200 dilution, and incubated with streptavidin-biotin peroxidase complex for 30 min. The slides were reacted with the substrate H_2O_2 in conjugation with diaminobenzidine (which stains brown) and with Mayer's hematoxylin (Sigma, St. Louis, MO) for nuclear counterstaining (which stains blue). Stained sections were examined on a Zeiss inverted microscope. The intensity of staining was recorded with arbitrary units ranging from − (no expression) to +++ (strongest expression). The technique was performed by the double-blinded method and thereby without knowledge of clinicopathological data.

RESULTS

Previously we showed that expression of cx43 was profoundly decreased in human brain tumor cell lines and that transfection of cx43 into human glioblastoma cell lines reverses the transformed phenotype in vitro and in vivo [13]. To determine whether the expression of cx43 was linked to the progression of human gliomas, we performed an immunohistochemical analysis in formalin-fixed, paraffin-embedded sections from human glioma tumors. The anti-cx43 antiserum that was generated, using the synthetic peptide corresponding to the cx43-specific sequence, specifically recognized the cx43 expressed in rat primary astrocyte and human fetal astrocyte cultures in Western blot assay [13]. To test the condition for the immunohistochemical staining, we used several dilutions of cx43 antibody to stain normal brain tissues. As shown in Figure 1A, a 1:200 dilution of cx43 antiserum strongly stained normal brain tissues containing glia and neurons, while the same dilution of preimmune serum did not produce any signal (Fig. 1B). Table I summarizes the immunohistochemical staining results. All three normal tissues showed positive immunoreactivity to cx43 antiserum (intensity arbitrary unit +++). In all grade I and grade II gliomas, immunoreactivity was also positive against cx43 antibody (+++). In contrast, in grade IV gliomas, no immunoreactivity to cx43 antibody was found in each of 6 samples examined (−), and in grade III gliomas, weak immunoreactivity against cx43 was found (− → +). Figure 1 shows cx43 immunoreactivity in sections from normal brain tissues (Fig. 1C), grade I (Fig. 1D), grade II (Fig. 1E), grade III (Fig. 1F), and grade IV (Fig. 1G) human gliomas. Normal human brain tissue and grade I and grade II gliomas show strong cx43 immunoreactivity, while grade III and grade IV gliomas show very little. More importantly, the expression of cx43 was specifically decreased in the adjacent area between benign and tumor tissue (Fig. 1H). These results suggest that expression of cx43 is decreased in high grades of human gliomas.

TABLE I. Expression of cx43 in Human Gliomas*

WHO grade	Pathology	cx43 level	No.
	Normal brain	+++	3/3
I II	Pilocytic astrocytoma Gemistocytic astrocytoma	+++	4/4
III	Anaplastic astrocytoma	− → +	8/8
IV	Glioblastoma multiforme	−	6/6

*The tissue sections were obtained from the Pathology Department, Northwest Hospital, Seattle, WA, and were stained with cx43 antiserum. The expression levels were determined by arbitrary unit from − (no expression) to +++ (strong expression) according to staining intensity. WHO, World Health Organization.

DISCUSSION

According to the World Health Organization (WHO), gliomas are classified into four major grades. Grade I (pilocytic astrocytoma) and Grade II (low-grade astrocytomas) are low-grade gliomas that usually grow slowly. Grade III (anaplastic astrocytoma) is a highly malignant glioma with increased cellularity, pleomorphism, and nuclear atypia. Grade IV is glioblastoma multiforme, which consists of poorly differentiated cells and spreads to other regions of the brain. These four grades represent the progression of gliomas. The expression of cx43 is reduced in grade III and grade IV, suggesting that the role of cx43 is not in the initiation of human gliomas, but that its reduced expression may perhaps affect the progression of the disease from low grades to high grades and more aggressive cancer. A number of studies support this model. Immunostaining studies indicate that human cx43 was highly expressed in normal mammary tissues, whereas the expression was decreased in the majority of primary tumors. Furthermore, no cx43 was expressed in metatastic tissues [8]. Similarly, normal human prostates accumulated high levels of cx43. In grade V prostate carcinoma, cx43 expression was greatly reduced. In grade III prostate carcinoma, cx43 expression is only moderately reduced [5]. The expression of cx43 was also specifically reduced in neoplastic skin lesions containing a squamous cell carcinoma but not in the papillomas [17]. The etiology of cancer is a complex multistep mechanism that has been defined to occur in three major steps: initiation, promotion, and progression. Reduction of gap junction communication often occurs at the promotion/progression stages of carcinogenesis. Many tumor promoters can disrupt gap junction communication

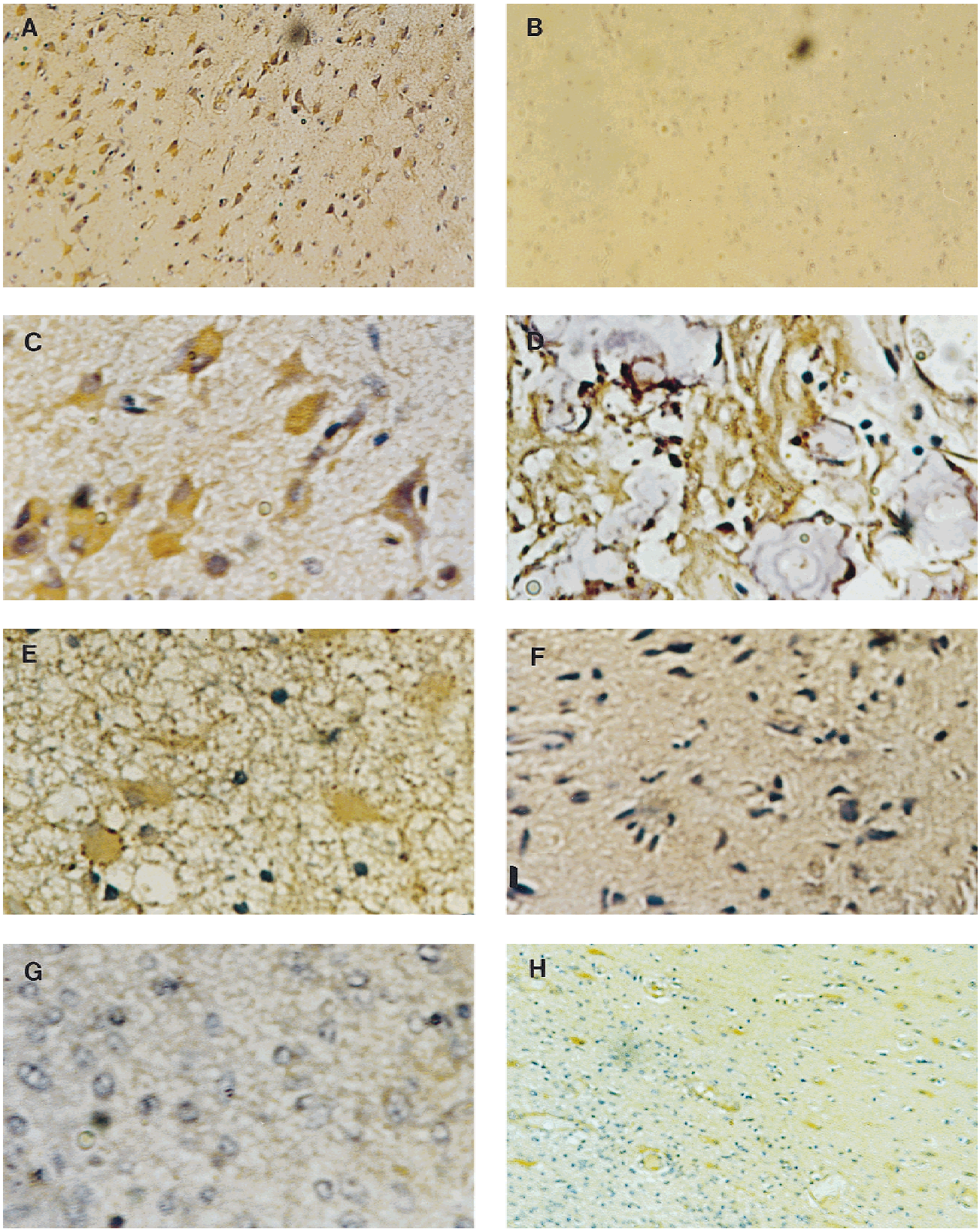

Fig. 1. Immunohistochemical analysis of human glioma tissues with anti-cx43 antiserum. Sections of formalin-fixed, paraffin-embedded tissues were stained with anti-cx43 antiserum (A,C–H) or preimmune serum (B). **A–C:** Benign tissue. **D:** Pilocytic astrocytoma. **E:** Gemistocytic astrocytoma. **F:** Anaplastic astrocytoma. **G:** Glioblastoma multiforme. **H:** Adjacent area between benign (left side) and tumor tissue (right side). ×1000 (A,B,H); ×400 (C–G).

(GJC) such as 12-O-tetradecanoylphorbol-13-acetate (TPA) [18,19], H_2O_2 [20,21], okadaic acid [22] (our unpublished data) and butylated hydroxytoluene [23]. These data suggest that reduced expression of cx43 is related to tumor development rather than initiation of tumorigenesis. Further studies are necessary in order to utilize cx43 as a biomarker of high grades of human gliomas.

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REFERENCES

1. Yamasaki H, Naus CCG: Role of connexin genes in growth control. Carcinogenesis 1996;17:1199–1213.
2. Beyer EC: Gap junctions. Int Rev Cytol 1993;137C:1–37.
3. Paul DL: New functions for gap junctions. Curr Opin Cell Biol 1995;7:665–672.
4. Wilgenbus KK, Kirkpatrick CJ, Knuechel R, et al.: Expression of cx26, cx32 and cx43 gap junction proteins in normal and neoplastic human tissues. Int J Cancer 1992;51:522–529.
5. Tsai H, Werber L, Davia MO, et al.: Reduced connexin43 expression in high grade, human prostatic adenocarcinoma cells. Biochem Biophys Res Commun 1996;227:64–69.
6. Lee S-W, Paul DL, Keyomarsi K, et al.: Transcriptional downregulation of gap junction proteins blocks junctional communication in human mammary tumor cell lines. J Cell Biol 1992;118: 1213–1221.
7. Tomasetto C, Neveu MJ, Daley J, et al.: Specificity of gap junction communication among human mammary cells and connexin transfections in culture. J Cell Biol 1993;122:157–167.
8. Hirschi KK, Xu CE, Tsukamoto T, et al.: Gap junction gene cx26 and cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. Cell Growth Differ 1996;7:861–870.
9. Zhu D, Caveney S, Kidder GM, et al.: Transfection of C6 glioma cells with connexin43 cDNA: Analysis of expression, intercellular coupling, and cell proliferation. Proc Natl Acad Sci USA 1991; 88:1883–1887.
10. Naus CC, Elisevich K, Zhou D, et al.: In vivo growth of C6 glioma cells transfected with connexin43 cDNA. Cancer Res 1992;52: 4208–4213.
11. Chen S-C, Pelletier DB, Ao P, et al.: Connexin43 reverses the phenotype of transformed cells and alters their expression of cyclin/cyclin-dependent kinases. Cell Growth Differ 1995;6:681–690.
12. Proulx AA, Lin Z-X, Naus CCG: Transfection of rhabdomyosarcoma cells with connexin43 induced myogenic differentiation. Cell Growth Differ 1997;8:533–540.
13. Huang RP, Fan Y, Hossain MZ, et al.: Reversion of the neoplastic phenotype of human glioblastoma cells by cx43. Cancer Res (in press).
14. Martyn KD, Kurata WE, Warn-Cramer BJ, et al.: Immortalized connexin43 knockout cells lines display a subset of biological properties associated with the transformed phenotype. Cell Growth Differ 1997;8:1015–1027.
15. Huang RP, Fan Y, De Belle I, et al.: Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. Int J cancer 1997;72:102–109.
16. Hossain MZ, Ao P, Boynton AL: Rapid disruption of gap junctional communication and phosphorylation of connexin43 by platelet-derived growth factor in T51B rat liver epithelial cells expressing platelet-derived growth factor receptor. J Cell Physiol 1998;174:66–77.
17. Sawey MJ, Goldschmidt MH, Risek B, et al.: Perturbation in connexin43 and connexin26 gap-junction expression in mouse skin hyperplasia and neoplasia. Mol Carcinog 1996;17:49–61.
18. Budunova IV, Williams GM: Cell culture assays for chemicals with tumor-promoting or tumor-inhibiting activity based on the modulation of intercellular communication. Cell Biol Toxicol 1994;10:71–116.
19. Yamasaki H: Non-genotoxic mechanisms of carcinogenesis: Studies of cell transformation and gap junctional intercellular communication. Toxicol Lett 1995;77:55–61.
20. Upham BL, Kang KS, Cho HY, et al.: Hydrogen peroxide inhibits gap junctional intercellular communication in glutathione sufficient but not glutathione deficient cells. Carcinogenesis 1997;18: 37–42.
21. Huang RP, Peng A, Fan Y, et al.: Tumor promotion by hydrogen peroxide in rat liver epithelial cell. Abstract no. 2534. In Proceedings of the 89th Annual Meeting of the American Association for Cancer Research, New Orleans, March 28–April 1, 1998:372.
22. Budunova IV, Carbajal S, Slaga TJ: Effect of diverse tumor promoters on the expression of gap-junctional proteins connexin (cx)26, cx31.1, and cx43 in SENCAR mouse epidermis. Mol Carcinog 1996;15:202–214.
23. Guan X, Hardenbrook J, Fernstorm MJ, et al.: Down-regulation by butylated hydroxytoluene of the number and function of gap junctions in epithelial cell lines derived from mouse lung and rat liver. Carcinogenesis 1995;16:2575–2582.